G-33-Y44
#1

Calpain Assays, Nerve injury and Repair

Annual Progress Report (G-33-Y44)

April 2001

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In the first year of this project, we have synthesized a number of new substrates for calpain I and II. The substrates were designed based on good transition-state inhibitors for calpain I. The following table gives representative hydrolysis data for a few of the substrates synthesized. The standard assay substrate for calpain I is Suc-Leu-Tyr-AMC (AMC = 4-methyl-7-aminocoumarin, a fluorescent leaving group). One of the substrates Ms-D-Ser(Bzl)-Phe-AMC (Ms = methanesulfonyl, Ser(Bzl) = O-benzylserine) is a 2.8 fold better substrate that the standard substrate Suc-Leu-Tyr-AMC.

		Rate [AMC (M)]/min * 10^{10}	
No.	Substrate[a]	Calpain I	Calpain II
-	Suc-Leu-Tyr-AMC	18.0	3.39
4	Z-Leu-Abu-AMC	10.7	3.28
5	Z-Leu-Leu-AMC	7.87	N.H.
6	Z-Leu-Nle-AMC	10.3	2.97
7	Z-Leu-Nva-AMC	15.3	5.65
8	Z-Leu-Phe-AMC	4.73	6.01
18	Ms-D-Ser(Bzl)-Phe-AMC	49.7	12.6
19	Ac-D-Ser(Bzl)-Phe-AMC	3.11	N.H.[b]

[a]41 μM of substrate used in assay, which is the solubility limit of almost all substrates. [b]N.H. denotes no hydrolysis.

We then measured K_{cat}/K_M values with calpain I and II for the Suc-Leu-Tyr-AMC and Ms-D-Ser(Bzl)-Phe-AMC substrates. The values are shown in the following table. The new substrate Ms-D-Ser(Bzl)-Phe-AMC was 1.8 fold better than the standard assay substrate Suc-Leu-Tyr-AMC and has a selectivity factor for calpain I vs calpain II of 4.6 compared to a value of 1.4 for Suc-Leu-Tyr-AMC.

k_{cat}/K_m ($M^{-1}s^{-1}$)			
Suc-Leu-Tyr-AMC		Ms-D-Ser(Bzl)-Phe-AMC	
Calpain I	Calpain II	Calpain I	Calpain II
31.8	22.6	57.7	12.35

Conclusion. We have prepared a new substrate for calpain I which is more reactive and more specific than the substrate in current use.

G-33-Y44
#2

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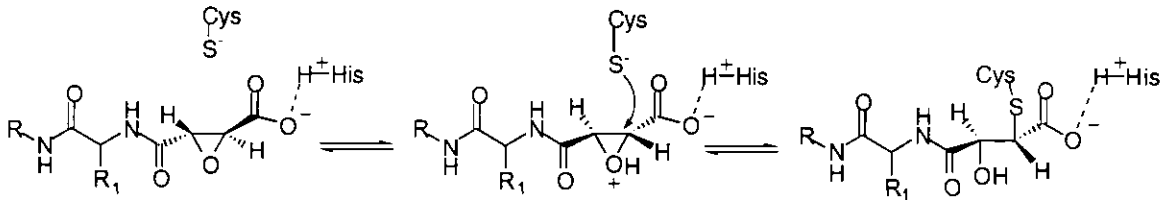
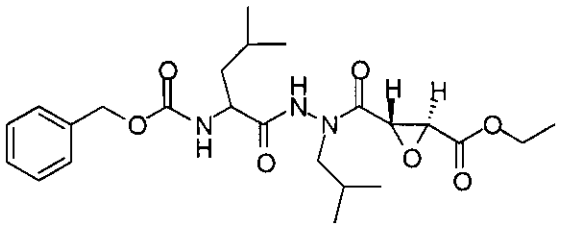
Progress Report. The Powers laboratory has recently developed a novel series of specific inhibitors for cysteine proteases based on the epoxysuccinate moiety. These inhibitors irreversibly inhibit cysteine proteases through reaction of the epoxide of the inhibitor with the active site cysteine residue of the target cysteine protease. During this year, we have developed specific epoxide inhibitors of calpain.

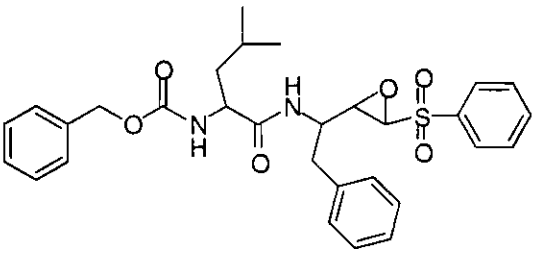
Epoxide inhibitors inhibit by forming a covalent bond with the cysteine residue in the protease active site (see the following figure).



Once the enzyme is bound to the epoxide no reverse reaction is possible and the enzyme is irreversibly inactivated. The incorporation of an aza-peptide moiety into the inhibitor structure allows for increased hydrogen bonding with the enzyme. A representative structure of an aza-peptide epoxide is shown in the following figure. An aza-peptide residue is an amino acid with a nitrogen substituted for the α-carbon. To be specific, we will refer to ALeu when the α-carbon of the amino acid leucine is replaced with nitrogen. The first structure (**1**) is a dipeptide aza-leucine derivative. The structure of a Leu-Phe dipeptide epoxysulfone (**10**) is also shown below. Epoxysulfones are derived from vinylsulfones, which are well known inhibitors of cysteine proteases. We will designate the epoxysulfone moiety as EPS.



1 Z-Leu-ALeu-EP (S,S)-COOEt



10 Z-Leu-Phe-EPS-Ph

Table 1. Inhibition of Calpain I by Aza-peptides and Epoxysulfones.[a]

No.	Inhibitor	k_{obs} ($M^{-1}s^{-1}$)
	Epoxides	
1	Z-Leu-ALeu-EP (*S,S*)-COOEt	8.58
2	Z-Leu-ALeu-EP (*R,R*)-COOEt	6.70
3	Z-Leu-ALeu-EP (cis)-COOEt	NI
4	Z-Leu-AHphe-EP (*S,S*)-COOEt	5.68
5	Z-Leu-AHphe-EP (*R,R*)-COOEt	2.16
6	Z-Leu-AHphe-EP (cis)-COOEt	1.03
7	Z-Leu-AHphe-EP (*S,S*)-COOH	2.12
8	EtOOC-EP (S,S)-Leu-NH-$(CH_2)_4$-NH-Z	1,770
9	HOOC-EP (S,S)-Leu-NH-$(CH_2)_4$-NH-Z	22,200
	Epoxysulfones	
10	Z-Leu-Phe-EPS-Ph	22.8
11	Z-Val-Phe-EPS-Ph	2.96

[a]ALeu = aza-leucine, AHphe = aza-homophenylalanine, Z = carboxybenzoyl.

Aza-peptide epoxides **1-7**, with the peptide on the left side of the epoxide functional group, show weak potency as calpain inhibitors. All of these inhibitors have a P2 Leu residue, which is preferred by calpain. We also investigated the role of epoxide stereochemistry on inhibitor potency. Both the *R,R* and *S,S* isomers at the epoxide moiety were equally potent. We then investigated epoxides **8** and **9**, which contain the peptide moiety on the right side of the epoxide functional group. These are more potent and indeed the most reactive derivative is **9**. These inhibitors will be used as lead compounds for the development of more potent and specific calpain inhibitors in the coming year. Epoxysulfones **10-12** are a new class of calpain inhibitors, which also show modest inhibition. We expect that epoxysulfones inhibit calpain by a mechanism similar to the mechanism of epoxide inhibition (see first figure). We plan to improve the inhibitor potency of epoxysulfones as calpain inhibitors during the year.

References

Bartus, R. T.; Elliott, P. J.; Hayward, N. J.; Dean, R. L.; Harbeson, S.; Straub, J. A.; Li, Z.; Powers, J. C. Calpain as a novel target for treating acute neurodegenerative disorders. *Neurological Research*. **1995**, *17*, 249-258.

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G-33-Y44
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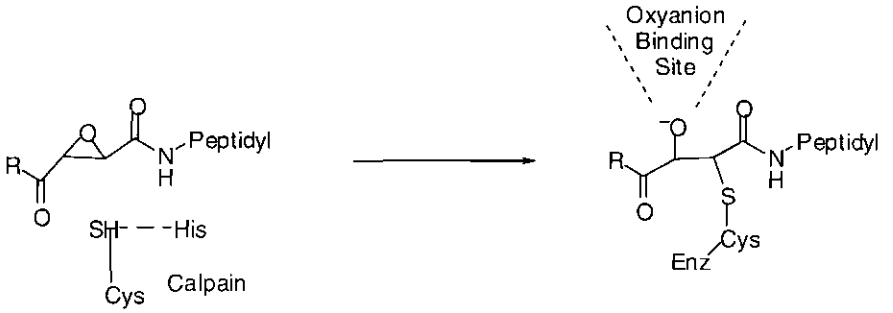
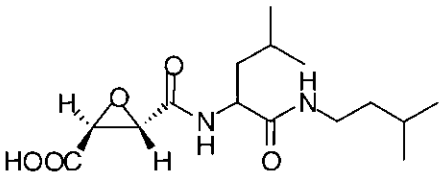
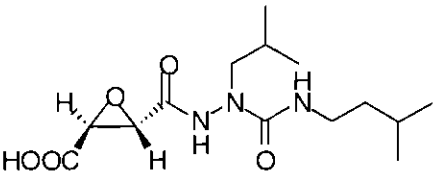
Progress Report. The Powers laboratory has recently developed a novel series of specific inhibitors for cysteine proteases based on the epoxysuccinate moiety. These inhibitors irreversibly inhibit cysteine proteases through reaction of the epoxide of the inhibitor with the active site cysteine residue of the target cysteine protease. Epoxide inhibitors inhibit by forming a covalent bond with the cysteine residue in the protease active site (see the following figure). During this year, we have developed specific epoxide inhibitors of calpain based on the structure of the natural inhibitor E-64c and incorporating aza-amino acids.



Once the enzyme is bound to the epoxide no reverse reaction is possible and the enzyme is irreversibly inactivated. The incorporation of an aza-peptide moiety into the inhibitor structure eliminates amino acid stereochemistry, changes the bond angles of amino acids, and provides new sites for possible hydrogen bonding to active sites. The structures of the natural epoxide E-64c and its aza-analog are shown in the following figure. An aza-peptide residue is an amino acid with a nitrogen substituted for the α-carbon. To be specific, we will refer to ALeu when the α-carbon of the amino acid leucine is replaced with nitrogen. We will designate the epoxysulfone moiety as EPS.



E64c (1)



Aza E64c (2)

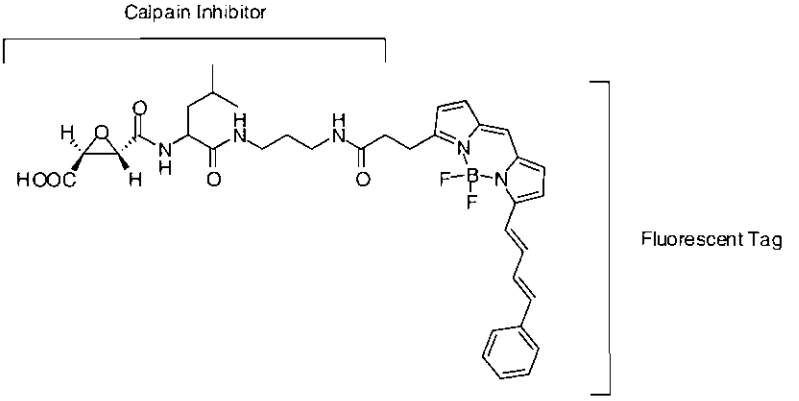
Table 1. Inhibition of Calpain I by Aza-peptide epoxides.[a]

No.	Inhibitor	k_{obs} ($M^{-1}s^{-1}$)
	Epoxides	
1	HO-EPS (S,S)-Leu-NH-$(CH_2)_2$-CH-$(CH_3)_2$ (E-64c)	17,200
2	HO-EPS (S,S)-ALeu-NH-$(CH_2)_2$-CH-$(CH_3)_2$	1,370
3	HO-EPS (S,S)-ALeu-NH-$(CH_2)_4$-NH-Z	1,450
4	HO-EPS (S,S)-ALeu-NH-benzyl	1220
5	HO-EPS (S,S)-ALeu-NH-$(CH_2)_3$-CH_3	840
6	HO-EPS (S,S)-ALeu-O-$(CH_2)_3$-CH_3	1000
7	HO-EPS (S,S)-ALeu-piperidine	1410

[a]EPS = epoxysuccinate, ALeu = aza-leucine, Z = carboxybenzoyl.

While the aza-peptide analogs of E64c are less potent than the parent compound, these inhibitors display significant inhibition of Calpain I. All of the best inhibitors contain the (S,S) epoxide stereochemistry and the carboxylic acid side chain. Epoxide **2** is the direct comparison to E64c (**1**). Inhibitor **3**, with a longer, aromatic side chain, shows a small improvement in potency. Epoxide **4**, with a shorter chain aromatic, is not at potent as epoxide **3**. In an attempt to increase the potency of epoxide **4**, with an amino-butyl side chain, the butyl ester analog **5** was synthesized. The ester allows for greater bond rotation, and does show an increase in potency. Epoxide **7** was also synthesized to improve bond rotation with the tri-substituted amine side chain, and displays an increase in potency.

Another ongoing project is the synthesis of a fluorescently-labeled inhibitor for calpain to be used as a diagnostic tool for the presence of calpain. The structure of the inhibitor in synthesis can be seen in the following figure. This inhibitor will be used to visualize the presence of calpain in cell cultures



References

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G-33-Y44
#4

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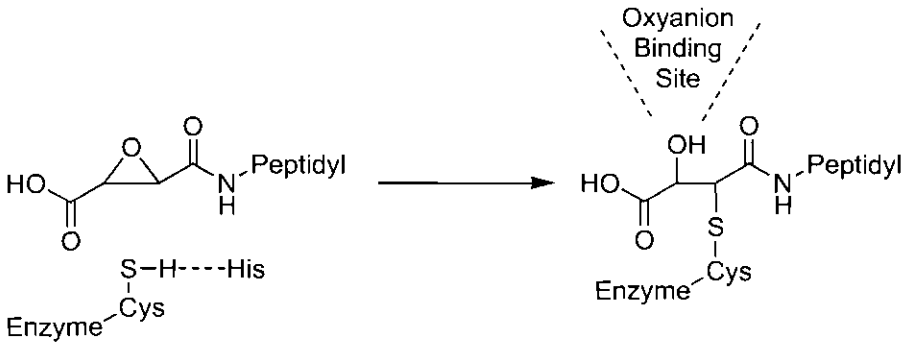
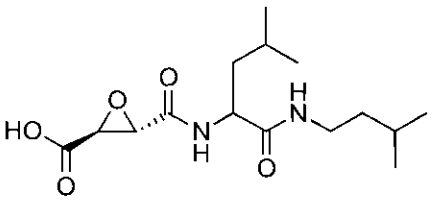
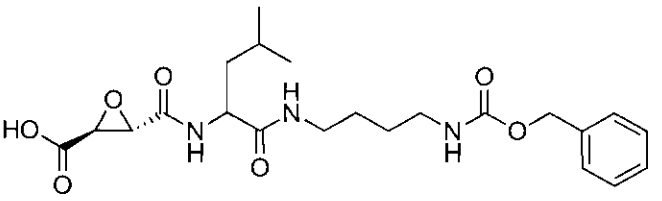


During this year, we have designed and synthesized a series of calpain inhibitors which are analogs of the epoxide inhibitor EP460. The structures of the natural epoxide E-64c and the most potent analog for calpain in the literature, EP460, are shown in the following figure. This year we have designed new analogs of EP460 substituting a variety of aromatics for the benzyl moiety in order to increase the potency and selectivity of the inhibitors for calpain and to gain information about the calpain active site.



E64c (1)



EP460 (2)

Table 1. Inhibition of Calpain I by peptide epoxides.[a]

No.	Inhibitor	k_{obs} ($M^{-1}s^{-1}$)
1	HO-EPS (S,S)-Leu-NH-$(CH_2)_2$-CH-$(CH_3)_2$ (E-64c)	7,200
2	HO-EPS (S,S)-Leu-NH-$(CH_2)_4$-NH-Z (EP460)	15,200
3	HO-EPS (S,S)-Leu-NH-$(CH_2)_4$-NH-CO-CH_2-O-1-naphthyl	21,600
4	HO-EPS (S,S)-Leu-NH-$(CH_2)_4$-NH-CO-NH-1-naphthyl	20,000
5	HO-EPS (S,S)-Leu-NH-$(CH_2)_4$-NH-CO-NH-benzyl	18,400
6	HO-EPS (S,S)-Leu-NH-$(CH_2)_4$-NH-CO-NH-2-phenoxyphenyl	15,600
7	HO-EPS (S,S)-Leu-NH-$(CH_2)_4$-NH-CO-NH-phenyl	13,400

[a]EPS = epoxysuccinate, Z = carbobenzyloxy.

The most potent epoxide inhibitors for calpain from our study are shown in Table1. EP460, the most potent epoxide inhibitor for calpain in the literature, is listed as inhibitor **2**. We were able to create several inhibitors that are more potent than EP460. Inhibitors **3** and **4** are the most potent inhibitors and both contain a naphthyl moiety. The naphthyl ring probably binds in a large hydrophobic pocket in the calpain active site. The benzyl urea inhibitor **5** which has an O to NH substitution shows greater potency than EP460. The phenoxyphenyl derivative **6** also shows promising potency. Inhibitor **7**, which is slightly less potent, demonstrates the importance of the methylene of the benzyl group for proper spacing in the calpain active site. Also, while these inhibitors showed increased potency for calpain, they showed decreasing potency for papain and cathepsin B compared to EP460. Therefore these inhibitors show increasing selectivity for calpain over papain and cathepsin B. Through this study, we have demonstrated that calpain accepts large aromatic groups near the S_3 subsite, and we have created the most potent epoxide inhibitor of calpain yet reported.

References

Bartus, R. T.; Elliott, P. J.; Hayward, N. J.; Dean, R. L.; Harbeson, S.; Straub, J. A.; Li, Z.; Powers, J. C. Calpain as a novel target for treating acute neurodegenerative disorders. *Neurological Research*. **1995**, *17*, 249-258.

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G-33-Y44
#5

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Final Progress Report (G-33-Y44)

September 2005

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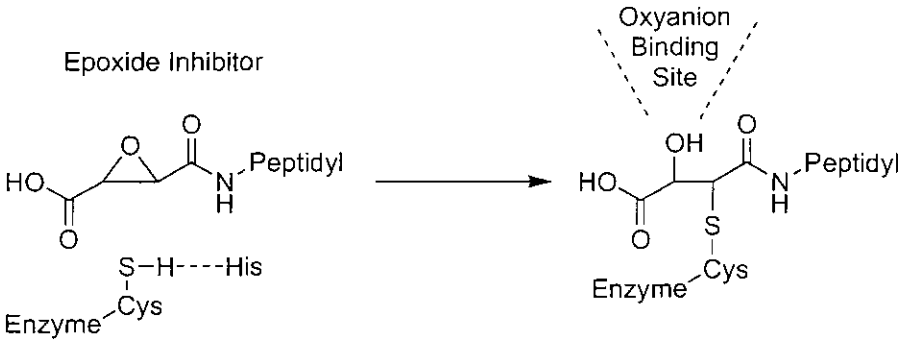
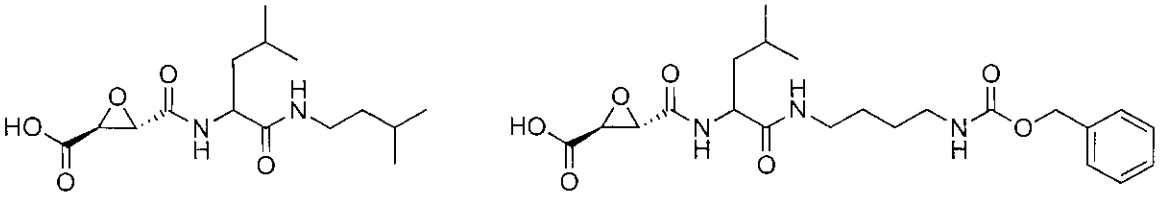


During this project, we have designed and synthesized a series of calpain inhibitors which are analogs of the epoxide inhibitor EP460 (**2**). The structures of the natural peptide epoxide E-64c (**1**) and the most potent inhibitor analog for calpain in the literature, EP460, are shown in the following figure. We have designed new analogs of EP460 by substituting a variety of aromatics for the benzyl moiety of EP460 in order to increase the potency and selectivity of the epoxide inhibitors for calpain and to gain information about the calpain active site.



E64c (1) **EP460 (2)**

Inhibition data for the new epoxides is shown in Table I.

Table 1. Inhibition of Calpain I by peptide epoxides.[a]

		$k_{obs}/[I]$ ($M^{-1}s^{-1}$)				% Inhibition of Calpain in
		Calpain I	**Calpain**	**Cathepsin**	**Papain**	**PC12 Cells**
	HO-EPS (S,S)-Leu-NH-CO_2-benzyl (EP460)	15,200		78,300	834,000	95
23a	EtO-EPS (S,S)-Leu-NH-CO_2-benzyl	500	1,520	120	510	90
24b	HO-EPS (S,S)-Leu-NH-CONH-benzyl	18,400	40,700	32,400	203,000	42
24c	HO-EPS (S,S)-Leu-NH-CONH-phenyl	13,400	41,200	36,000	308,000	ND
24d	HO-EPS (S,S)-Leu-NH-CONH-(4-methoxyphenyl)	11,500	28,300	30,000	387,000	73
24e	HO-EPS (S,S)-Leu-NH-$COCH_2$-(3-pyridyl)	3,080	8,060	19,500	188,000	ND
24f	HO-EPS (S,S)-Leu-NH-$COCH_2O$-(1-naphthyl)	21,600	46,500	42,500	232,000	81
24g	HO-EPS (S,S)-Leu-NH-$COCH_2O$-(2-naphthyl)	12,400	20,200	17,600	194,000	ND
24h	HO-EPS (S,S)-Leu-NH-CONH-(1-naphthyl)	20,000	44,000	45,100	666,000	70
24i	HO-EPS (S,S)-Leu-NH-CONH-(2-phenoxyphenyl)	15,600	39,500	57,300	651,000	84

ND = not determined; EPS = epoxysuccinate.

All of the inhibitors in Table 1 are irreversible inhibitors with second order inhibition rate constants ($k_{obs}/[I]$) with calpains as high as 46,000 $M^{-1}s^{-1}$. Many of the inhibitors synthesized were more potent with calpain I and II than EP460. The best inhibitors for the calpains incorporated the 1-naphthyl moiety (**24f**, **24h**). The benzyl urea compound (**24b**) and the phenoxyphenyl urea (**24i**) were also potent inhibitors. The EP460 analog containing an ethyl ester (**23a**) on the epoxysuccinate moiety was a significantly less potent inhibitor with all of the enzymes, demonstrating the importance of the terminal epoxide carboxylic acid for inhibitor binding. Generally, the best inhibitors for calpain I and calpain II had the same structure, however the inhibitor potency with calpain II was 2-fold greater than calpain I. It is worth noting that while some of the inhibitors demonstrated greater inhibitory potency with calpain than EP460, all of the new inhibitors are significantly less potent than EP460 with cathepsin B and papain. A few of the inhibitors (**24b**, **24c**, **24f**) are even more potent with calpain II than with cathepsin B. Thus, the design changes had increased the specificity of the inhibitors for calpain vs cathepsin B. The inhibitors also inhibited the cysteine protease papain with high rate constants. Since papain is not a human protease, selectivity over papain is inconsequential for our purposes of drug development. In summary, we have succeeded in the discovery of new epoxide calpain inhibitors that are more potent and selective than EP460.

The best of these inhibitors were also tested in cells for their ability to inhibit calpain activity in PC12 cells, a breast cancer cell line, induced by the chemotherapy drug taxol by Dr. Jonathan Glass at Emory University. One of the side effects of the drug taxol in humans is peripheral neuropathy, where patients experience tingling and loss of sensation in the limbs. Dr. Glass has demonstrated that the addition of taxol to PC12 cells spikes the calpain proteolytic activity present in the cells. These results indicate that calpain is involved in the mechanism by which taxol induces peripheral neuropathy. One of the many therapeutic possibilities of calpain inhibitors such as these epoxides is to treat this side effect of taxol.

The results of this cell study can be seen in Table 1. The results are reported as % inhibition of calpain relative to the amount of spiked calpain activity with taxol alone. Several of the epoxide inhibitors proved to be more potent calpain inhibitors than the α-ketoamide AK295, a hallmark calpain inhibitor, in this assay. EP460 (**24a**) is the most potent inhibitor in this assay, along with the ethyl ester derivative **23a**. Although the **23a** is a much weaker inhibitor *in vitro* studies, the ethyl ester is hydrolyzed by esterases in cells, generating the more potent acid form **24a**. It is therefore reasonable that **23a** and **24a** have differing potencies *in vitro*, but inhibit similarly in cells. The methoxyphenyl and phenoxyphenyl inhibitors **24d** and **24i** were the most potent of the new inhibitors in this assay. It is interesting to note that **24d** and **24i** were not the most potent *in vitro*. It seems that these inhibitors are more bioavailable and better able to cross the cell membrane to reach the calpain. This experiment demonstrates that these epoxide inhibitors are able to cross cell membranes and efficiently inhibit calpain in a cell culture, which is an important step toward their development as therapeutic agents.

References

Bartus, R. T.; Elliott, P. J.; Hayward, N. J.; Dean, R. L.; Harbeson, S.; Straub, J. A.; Li, Z.; Powers, J. C. Calpain as a novel target for treating acute neurodegenerative disorders. *Neurological Research*. **1995**, *17*, 249-258.

Saatman, K. E.; Murai, H.; Bartus, R. T.; Smith, D. H.; Hayward, N. J.; Perri, B. R.; McIntosh, T. K. Calpain Inhibitor AK295 Attenuates Motor and Cognitive Deficits Following Experimental Brain Injury in the Rat. *Proc. Natl. Acad. Sci. USA*. **1996**, *93*, 3428-3433.

Publications

None yet

Patents

None